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Purification and Properties of Histidinol Dehydrogenases from Psychrophilic, Mesophilic and Thermophilic Bacilli

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As a first step in elucidating one molecular mechanism of adaptation to life at extreme temperatures, we purified and characterized the enzyme histidinol dehydrogenase (EC 1.1.1.23) from a number of bacilli whose growth temperatures range from 5°t o 90°C. The enzymes were purified by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on Sephadex, affinity chromatography on histamine- or histidine–Sepharose and preparative gradient gel electrophoresis. All had similar mol.wts. (29200), sedimentation coefficients ($s_{20,w}$ 2.56S), affinities for histidinol and NAD^+ ($K_m = 48\,\mu M$ and 0.2 mM respectively) and all had pH optima at 9.6. Marked differences were observed in stability with respect to temperature and the temperature at which the initial velocity for histidinol dehydrogenation was optimal. These optima range from 25°C for the enzyme from the psychrophilic species through to 41°C for the mesophiles to 85–92°C for the extreme thermophiles. It is concluded that the ability of the enzymes to operate at their various optimum temperatures is an intrinsic property of their amino acid sequences.

We are interested in the mechanisms that organisms have evolved for their enzymes to operate under a variety of environmental and physiological conditions. For this purpose we chose the enzyme histidinol dehydrogenase (EC 1.1.1.23), which is the terminal enzyme in the biosynthetic pathway of histidine formation and is essential for the existence of all organisms which do not rely on a supply of histidine in the environment. By using this enzyme as a model system it has previously been shown that a diversity of strategies have been evolved to control its operation. In *Salmonella* and related bacteria the enzyme is produced as part of the activities of an operon system, its formation being under strict control. Histidinol dehydrogenase from *Salmonella typhimurium* has been characterized by Loper (1968) and Yourno & Ino (1968) and is a dimer of identical subunits, each of mol.wt. 40000. In *Neurospora* the enzyme is a multifunctional protein, catalysing two other steps in the histidine pathway and is of 135000 mol.wt. (Minson & Creaser, 1969). In *Arthrobacter histidinolovorans*, which can use histidinol as a sole source of carbon and nitrogen (Adams, 1954), two enzymes can be produced, both specific for the dehydrogenation of histidinol; one is the normal biosynthetic enzyme, the second is only produced when histidinol is present in the medium (Dhawale *et al.*, 1972).

We are seeking to extend our work to investigate the enzyme histidinol dehydrogenase in organisms that are adapted to environmental extremes, with special reference to thermophily. Several possible mechanisms (Singleton & Amelunxen, 1973) have been suggested to enable enzymes to operate at elevated temperatures. These include: greatly increased synthesis of the enzyme to offset increased thermal degradation; stabilization of the enzyme structure by non-protein factors or bivalent metal ions, or by association with membranes; and the synthesis of enzymes that are intrinsically thermostable owing to their sequence and configuration. It seems unlikely that there is a single strategy used by all organisms. To date most of the experiments on the thermostability of enzymes has been done on a comparative basis, contrasting the properties of the same enzyme derived from mesophilic and quite often unrelated thermophilic organisms. We have used a group of closely related bacteria on the premise that such differences that we may observe stand more chance of being correlated with the different temperature stabilities of the enzyme rather than due to large taxonomic divergency.

We have examined the histidinol dehydrogenase enzyme in a variety of bacilli that have grown temperatures from 5° to 90°C, using these definitions: thermophiles, organisms whose optimum growth temperature is above 65°C (Farrell & Campbell, 1969); psychrophiles, organisms having an optimum growth temperature of about 15°C and a maximum growth temperature about 20°C (Morita, 1975);

mesophiles, those organisms whose optimum growth temperatures lie between those temperatures defined for psychrophiles and thermophiles (i.e. 20–65°C).

Materials and Methods

All chemicals were of AR quality; L-histidine, NAD^+, Tris, MTT tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and bovine serum albumin were preparations of Sigma Chemicals, St. Louis, MO, U.S.A.; phenazine methosulphate was obtained from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; nutrient broths, agars and yeast extracts were obtained from Difco Laboratories, Detroit, MI, U.S.A., and Oxoid Division of Oxo, London E.C.4, U.K. Brain/heart infusion was a product of Oxoid. All Sephadex and Sepharose gels were from Pharmacia, Uppsala, Sweden. Gradipore acrylamide gels were obtained from Gradient Pty. Ltd., Sydney, Australia.

Bacillus species

The following species were used in this work; their origin and optimum growth temperature are enumerated: *Bacillus psychrophilus* A.T.C.C. 23304, 15°C; *Bacillus subtilis* WB2802, from Dr. E. W. Nester, 37°C; *Bacillus stearothermophilus* A.T.C.C. 12980, 55°C; *Bacillus subtilis* HT-1, obtained by transformation (Lindsay & Creaser, 1975), 72°C; *Bacillus caldolyticus*, from Dr. E. D. MacElroy, 72°C.

Growth media

Strains of bacilli were stored as suspensions in either 7.5% (v/v) dimethyl sulphoxide or 30% (v/v) glycerol at −20°C. Continuous cultures were also kept, by weekly subculture of all strains and storing them at 4°C. Liquid media were prepared as follows and autoclaved at 108kPa (15lb/in^2) for 20min before use. For *B. psychrophilus*: glucose, 5g; yeast extract, 5g; KH_2PO_4, 6g; K_2HPO_4, 14g; $(NH_4)_2SO_4$, 2g; water, 1 litre. For *B. subtilis*: glucose, 5g; yeast extract, 5g; bactotryptone, 5g; NaCl, 5g; water, 1 litre. For *B. stearothermophilus*: minimal salts medium, 1 litre (Spizizen, 1958); glucose, 10g; yeast extract, 5g; for *B. caldolyticus* and *B. subtilis* HT-1: glucose, 3g; nutrient broth no. 2, 2.5g; brain/heart infusion, 2.5g; NH_4NO_3, 0.5g; KH_2PO_4, 0.5g; NaCl, 0.25g; $MgSO_4$, 0.25g; $FeSO_4$, 0.05g; $CaCl_2$, 0.05g; water, 1 litre; micronutrient solution (Heinen, 1971), 0.15ml; the pH adjusted to 7.8 with Na_2SiO_3.

Determination of protein

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme assay

L-Histidinol dehydrogenase activity was measured by NAD^+ reduction as the rate of increase in A_{340} at 37°C (Creaser *et al.*, 1967). A spectrophotometer cuvette (10mm light-path) contained 0.7ml of 0.05M-glycine/NaOH buffer, pH9.8, 0.1ml of NAD^+ (1μmol) and 0.1ml of the enzyme under test. The mixture was incubated for 2min at the various optimum temperatures required for the histidinol dehydrogenases from each *Bacillus* species, and then 0.1ml (0.2μmol) of L-histidinol dihydrochloride solution was added to initiate the reaction. NAD^+ reduction was then measured. Enzyme activities are expressed in nkat, which indicates formation of 1nmol of reduced substrate (NADH)/s.

Affinity chromatography

Preliminary experiments (Lindsay *et al.*, 1974) showed that the substrate L-histidinol and the inhibitors histamine and L-histidine would make the best affinity adsorbents. Three affinity columns were prepared, the general design being Sepharose–C_6 spacer–L-histidinol, L-histidine (attached via its carboxyl group) or histamine.

Sepharose 4B or 6B was used as supplied by Pharmacia. Swollen gel (500ml) was equilibrated with a solution of 20g of CNBr in 500ml of water, for 1h at 25°C. The solution was adjusted to pH11.5 and kept for 10min by the addition of 5M-NaOH with vigorous stirring. The gel was filtered off and washed extensively with ice-cold water, followed by 0.5M-Na_2CO_3 at 4°C. This CNBr–Sepharose was immediately treated with spacer (50g) at pH8.5 for 15h at 25°C. Spacers used were either 1,6-diaminohexane or 6-aminohexanoic acid. The method was based on the procedure of Axen *et al.* (1967). The spacer–Sepharose was treated with the ligands, L-histidine with the aminohexane adsorbent and histamine or L-histidinol with the hexanoic acid adsorbent: 30ml of spacer–Sepharose was mixed with 1g of ligand in 18ml of water, then 1g of ethyl-3-dimethylaminopropylcarbodi-imide was added. The pH was kept at 4.5 for 15h with 2.0M-HCl, then the adsorbent was filtered off and washed successively with 0.5M-NaCl, 0.01M-HCl, then equilibrated with 0.05M-Tris/HCl buffer, pH9.15.

Chromatographic purification by using the affinity gels was performed in a 2cm×25cm column. Each gel was cleaned and equilibrated before use by sequential elution with 100ml of 0.5M-NaCl, pH10.5, then 100ml of 1mM-HCl, then 100ml of 0.5M-NaCl, pH5.5, and finally 100ml of 0.05M-Tris/HCl buffer, pH9.15.

Gradient electrophoresis

For detection of enzyme activity after electrophoresis, Gradipore polyacrylamide gels were incubated at the appropriate specific optimum tempera-

tures in the following solution: 1.0ml of L-histidinol dihydrochloride solution (1mg/ml), 0.2ml of NAD^+ solution (20mg/ml), 0.2ml of MTT tetrazolium solution (1mg/ml), 0.2ml of phenazine methosulphate solution (1mg/ml) and 20ml of 0.05M-Tris/HCl buffer, pH9.15.

The method for staining for histidinol dehydrogenase activity is based on the ability of the tetrazolium salt to accept an electron through the agency of phenazine methosulphate (Nachlas *et al.*, 1960), causing the development of a blue colour.

For protein, gels were stained with 0.1% Amido Black in 7% (v/v) acetic acid, and destained with water/methanol/acetic acid (20:5:3, by vol.).

Results

Purification of histidinol dehydrogenases

General procedure. Step. 1. Large-scale batches of cells of the different *Bacillus* species were grown at their optimum temperature in their specific medium. The cells were harvested at the end of exponential phase by centrifugation at 40000rev./min in a Sharples Superspeed centrifuge. The cells (approx. 2.0–2.5g wet wt./litre) were resuspended in 0.05M-Tris/HCl buffer, pH9.15, and treated with 50mg of lysozyme (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) and 5mg of deoxyribonuclease (Calbiochem, San Diego, CA, U.S.A.) for 30min at 37°C. The cells were further disrupted by sonication for 5min in an MSE 100W Ultrasonic disintegrator on a setting of 5μm. The suspension was then centrifuged at 6000*g* for 20min in a Sorvall RC2B centrifuge, and the cell precipitate was discarded.

Step. 2. The supernatant was treated with 0.05vol. of 1M-$MnCl_2$ to remove the nucleic acids, centrifuged at 6000*g* for 20min and the precipitate discarded.

Step 3. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant to give 70% saturation and the mixture was left stirring at 3°C for 4h. The suspension was then centrifuged at 6000*g* for 20min, the precipitate collected and resuspended in a minimal volume of 0.05M-Tris/HCl buffer, pH9.15. This solution was then dialysed for 18h against 20vol. of the same buffer to remove $(NH_4)_2SO_4$.

Step 4. The crude extract was then heated for 10min at 10°C above the optimum growth temperature for that *Bacillus* strain and was then centrifuged at 27000*g* for 10min to remove precipitated protein. The supernatant was collected, concentrated in an Amicon Diaflo system with a UM-2 membrane, and redialysed against 0.05M-Tris/HCl buffer, pH9.15, any resulting precipitate being discarded after centrifugation.

Step 5. Purification by affinity chromatography was performed by adsorbing the heated crude extract on the appropriate affinity column, previously regenerated by the standard method. Factors that determine the choice of an appropriate column are discussed under 'Specific procedures'. The non-attached protein was washed off with 0.05M-Tris/HCl buffer, pH9.15. The absorbed proteins, including histidinol dehydrogenase, were eluted with 0.1M-histidine, pH9.15. The active eluates were collected, concentrated and dialysed against 0.05M-Tris/glycine buffer, pH8.3, and stored at 4°C. After each enzyme purification the gel itself must be cleaned as described in the Materials and Methods section. The quantity of protein that may be loaded on the column is also limiting, owing to the binding of unwanted proteins. This non-specific binding may be decreased, however, by pre-equilibrating the column with 1mM-KCl adjusted to pH9.15 with 1M-NaOH.

Step 6. Final purification was performed by preparative gel electrophoresis. Samples (1.5ml) were loaded on Gradipore polyacrylamide gels and left to run overnight at 3°C (5mA/gel) in 0.05M-Tris/glycine buffer, pH8.3. The gels were removed and stained for their histidinol dehydrogenase activity. The active bands were cut out and the enzyme was extracted by breaking the gel in a Waring blender in 0.05M-Tris/glycine buffer, pH8.3. The preparation was then centrifuged at 30000*g* for 30min, after which the supernatant was removed and dialysed against water. Samples were stored at −20°C for further analysis.

This procedure resulted in enzyme preparations giving coincident protein and activity bands in Gradipore polyacrylamide gels at pH8.3, and single components in the analytical centrifuge. A representative purification (*B. stearothermophilus*) is shown in Table 1, and a summary in Table 2.

Table 1. *Purification of histidinol dehydrogenase from B. stearothermophilus*

Purification step	Volume (ml)	Total protein (mg)	Specific activity (nkat/mg of protein)	Total activity (nkat)	Recovery (%)
Crude extract	280	14000	0.067	938	
$(NH_4)_2SO_4$ ppt. (dialysed)	105	2890	0.294	850	91
Heating	105	1280	0.66	840	89
Affinity column	40	53	15.64	829	85
Preparative gel	16	8	64.75	518	58

Table 2. *Summary chart for histidinol dehydrogenase purification*

Bacillus	Enzyme extract	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)
B. psychrophilus	Crude	9000	290	0.03
	Pure	2.1	109	51.9
B. subtilis	Crude	11150	12500	1.12
	Pure	29.7	1762	59.33
B. stearothermophilus	Crude	14000	938	0.067
	Pure	8	518	64.75
B. caldolyticus	Crude	12000	1970	0.16
	Pure	20	1296	64.80
B. subtilis HT-1	Crude	12800	2333	0.182
	Pure	25	1620	64.80

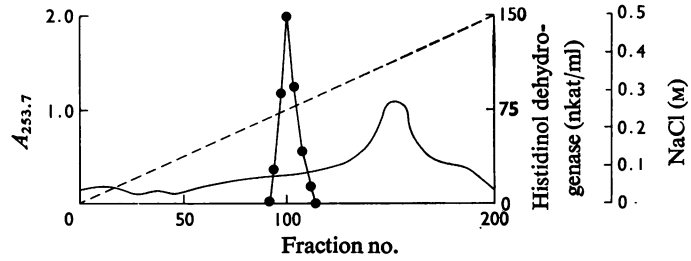

Fig. 1. *Chromatography of histidinol dehydrogenase on a column* (100 cm × 2 cm) *of QAE-Sephadex A-25*
Elution was by a gradient (----) of 0–0.05 M-NaCl in 0.01 M-phosphate buffer, pH 6.8, and the protein trace (——) was recorded with a LKB Uvicord instrument. Histidinol dehydrogenase activity (●) was measured by NAD^+ reduction. Fractions (10 ml) were collected.

Specific procedures. There were problems with the purification procedure with regard to the *B. subtilis* WB2802 histidinol dehydrogenase enzyme. Since the strain is de-repressed for several biosynthetic pathways, including that for histidine, a larger amount of unwanted protein would be expected. In practice an excess of protein was observed and this decreased the binding of the histidinol dehydrogenase enzyme to the affinity column. Hence it was necessary to include another purification step before application to the affinity column. This step entailed the use of a column (100 cm × 2 cm) of QAE (quaternary aminoethyl)-Sephadex A-25, equilibrated with 0.01 M-sodium/potassium phosphate buffer, pH 6.8. A 0–0.5 M-NaCl gradient in the equilibration buffer was used to elute the enzyme. This step removed a large quantity of unwanted protein (Fig. 1).

One of the affinity columns tested, the Sepharose–spacer–histidinol adsorbent, would bind all the histidinol dehydrogenase enzymes tested. However, the life of these columns was short, presumably owing to the bound histidinol being reduced by the enzyme because of trace amounts of NAD^+ in the preparations. However, both the histidinol dehydrogenase enzymes from *B. psychrophilus* and *B. subtilis* could be purified on histamine affinity columns and those from *B. subtilis* HT-1, *B. stearothermophilus* and *B. caldolyticus* could be purified with the histidine column. The active enzymes from the latter three organisms were eluted at high temperatures, that from *B. stearothermophilus* at 55°C and those of *B. caldolyticus* and *B. subtilis* HT-1 at 70°C. *B. subtilis* and *B. psychrophilus* enzymes were eluted at 25°C. When the histamine column was used, the eluent to desorb the histidinol dehydrogenase was 1 M-imidazole, pH 9.1.

Enzyme properties

Ultracentrifugation. Ultracentrifugation studies were carried out in an MSE Centriscan 75 analytical centrifuge. Samples from the last stage of purification were used; they had previously been dialysed overnight against 0.05 M-Tris/HCl buffer, pH 9.15. Samples were spun at various speeds and protein concentations to achieve maximum accuracy for both s value and molecular-weight determinations.

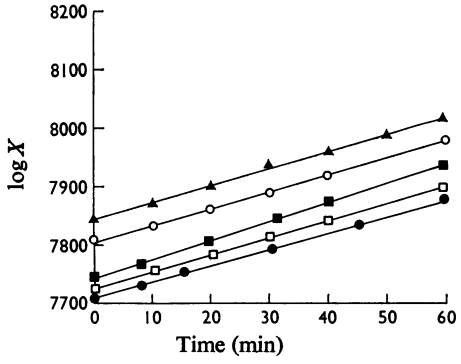

Fig. 2. *Sedimentation of histidinol dehydrogenase enzymes from Bacillus spp.*
log X, where X is the distance of the boundary from the centre of rotation, was plotted against time for histidine dehydrogenases from the following species: ●, *B. psychrophilus*; □, *B. subtilis*; ▲, *B. stearothermophilus*; ○, *B. caldolyticus*; ■, *B. subtilis* HT-1. $s_{20,w}$ values were calculated from the gradient by the method of Schachman (1957). Protein concentration was 1 mg/ml in all cases.

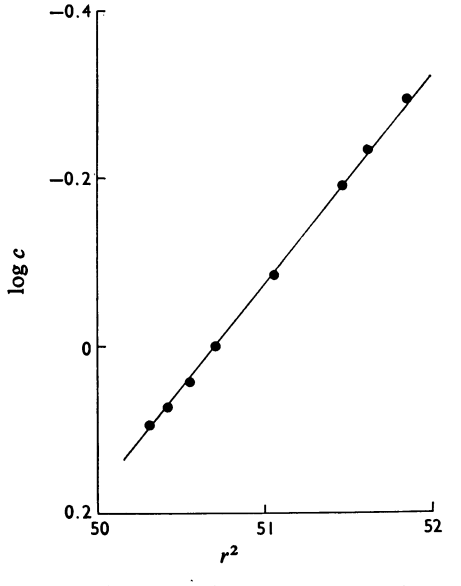

Fig. 3. *Molecular-weight determination, by sedimentation equilibrium, of histidinol dehydrogenase from B. stearothermophilus*
Centrifugation was performed at 20°C, at 18500 rev./min for 24h. log c (the protein concentration at a given point) was plotted against r^2 (distance from the centre of rotation), the slope being proportional to the molecular weight.

Table 3. *Summary of molecular weights and $s_{20,w}$ values for histidinol dehydrogenases from Baccillus spp.*

	$s_{20,w}$ (S)	Mol.wt.
B. psychrophilus	2.6	29250
B. subtilis	2.5	29000
B. stearothermophilus	2.5	29000
B. caldolyticus	2.6	29000
B. subtilis HT-1	2.6	29700

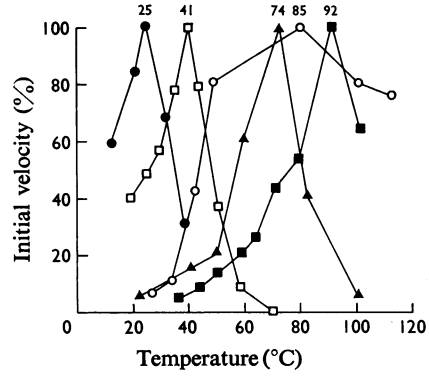

Fig. 4. *Effect of temperature on initial velocity of histidinol dehydrogenases from Bacillus spp.*
The rate of NAD^+ reduction at various temperatures was measured and the rates obtained were plotted against temperature. Optima are indicated on the Figure. Species key is as in Fig. 2.

Molecular-weight determinations for the various proteins were calculated by the sedimentation-equilibrium method (Schachman, 1957). The conditions for attaining equilibrium were centrifugation at 18500 rev./min for 24h at 20°C. Protein samples were of 1 mg/ml concentration, in 0.05 M-Tris/HCl buffer, pH 9.15.

Calculations for the molecular weights were based on the procedure of Schachman (1957), assuming a partial specific volume of 0.72, and a density of 1.0. Fig. 2 shows the sedimentation analysis for all enzymes and Fig. 3 shows a representative molecular-weight determination (*B. stearothermophilus*). All s values and molecular weights are summarized in Table 3.

Effects of temperature. The initial activities of the enzymes were determined in the temperature range 5–100°C, by using a Pye–Unicam SP.1800 spectrophotometer linked to a recirculating Haake water bath. Fig. 4 shows the initial activity curves for all histidinol dehydrogenase enzymes. The optimum temperatures for histidinol dehydrogenase activity

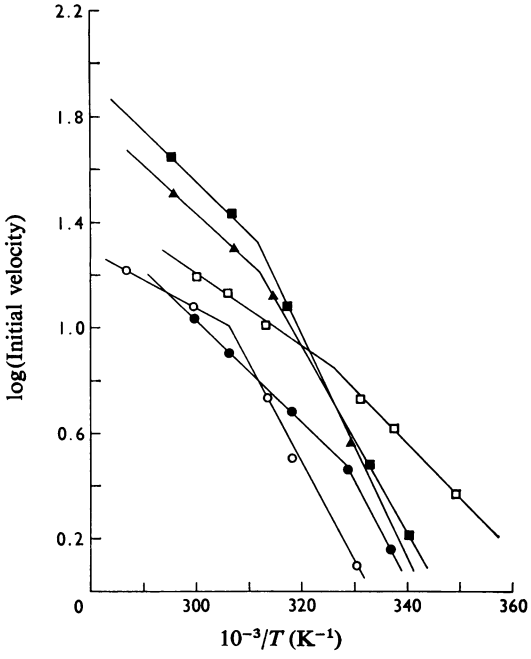

Fig. 5. *Arrhenius plot for histidinol dehydrogenase enzymes from Bacillus spp.*
log(Initial velocity) was plotted against the reciprocal of the absolute temperature. Temperatures of discontinuities are: ●, 32°C; □, 35°C; ▲, 47°C; ■, 48°C; ○, 52°C.

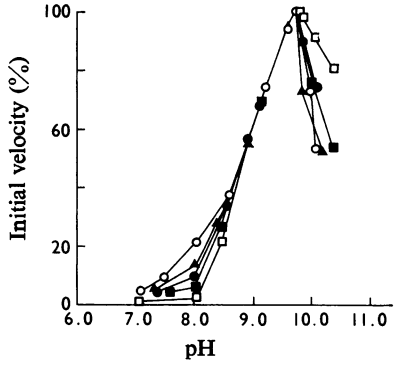

Fig. 6. *Effect of pH on histidinol dehydrogenase from Bacillus spp.*
Initial velocity was plotted against pH, obtained from various buffers as detailed in the text. Species key is as in Fig. 2.

were as follows: *B. psychrophilus*, 25°C; *B. subtillis*, 41°C; *B. stearothermophilus*, 74°C; *B. caldolyticus*, 87°C; *B. subtilis* HT-1, 92°C.

Arrhenius plots. Arrhenius plots were performed as described in Dixon & Webb (1964). The initial velocities were studied from 15 to 85°C. The negative slope is an expression of activation energy E expressed as J/mol (Fig. 5).

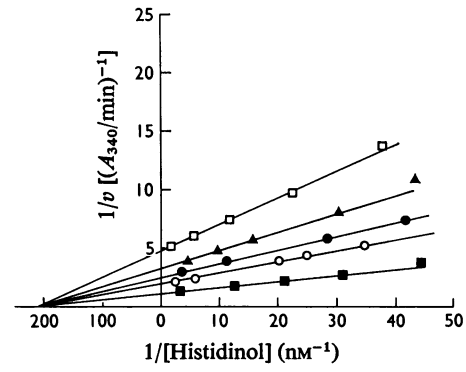

Fig. 7. *Double-reciprocal plot to determine K_m (histidinol) of histidinol dehydrogenases from Bacillus spp.*
NAD^+ concentration was saturating. Key is as in Fig. 2.

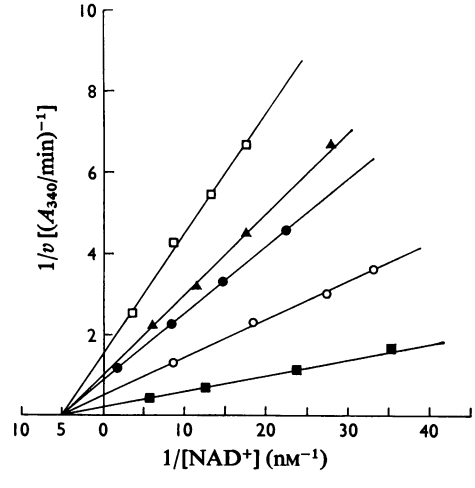

Fig. 8. *Double-reciprocal plot to determine K_m (histidinol) of histidinol dehydrogenases from Bacillus spp.*
Histidinol concentration was saturating. Key is as in Fig. 2.

Effects of pH (Fig. 6). To determine the optimum pH for the reaction catalysed by histidinol dehydrogenase, activities were determined at the various optimum temperatures. Buffers (0.05 M) were glycine/NaOH, pH8.6–10.4, Tris/HCl, pH7.2–8.2, and sodium/potassium phosphate buffer at pH6.8.

Michaelis constants. The Michaelis constants K_m (histidinol) and K_m (NAD^+) were determined at the

optimum reaction temperature for each enzyme by double-reciprocal plots (Lineweaver & Burk, 1934). All plots of $1/v$ against $1/s$ were linear over concentration ranges 8–80 μM (histidinol) and 40–400 μM (NAD^+). These results gave a K_m for NAD^+ of 0.2 mM and a K_m for histidinol of 48 μM. The concentration of NAD^+ was saturating when histidinol was the variable (Fig. 7), and the concentration of histidinol was saturating when NAD^+ was varied (Fig. 8).

Discussion

The five histidinol dehydrogenase enzymes tested were very similar. Their sedimentation coefficients, molecular weights, optimum pH and Michaelis constants for both substrates (histidinol and NAD^+) were identical. However, the temperatures of optimum activity were strikingly different and correlated with the growth temperatures of the organisms from which they were purified. Arrhenius plots showed similar but not identical graphs, with one concavity and one discontinuity. The temperatures where the discontinuities occurred could be arranged in a progression (32°, 35°, 47°, 48°, 52°C) approximately correlating with the increasing temperature optima of the histidinol dehydrogenases from the various thermally adapted organisms.

The enzymes were all pure; no evidence was obtained of any stabilizing factors not part of the pure enzymes. The curves relating temperature to optimum initial activity were identical in both pure and crude preparations.

It is noteworthy that identical Michaelis constants were found at the optimum temperature of initial velocity, even though their optima were markedly different. One can presume that the actual active sites are very similar, but that during evolution the structure of the histidinol dehydrogenase polypeptide chains have been changed to conserve the character of this active site in the temperature-adapted bacilli.

We conclude that the different responses to temperature of the histidinol dehydrogenases tested are functions of their different primary amino acid sequences and configurations.

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